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(71) Applicant: CELL THERAPEUTICS, INC. [US/US]; Suite 400, 201 Elliott Avenue West, Seattle, WA 98119 (US).

(72) Inventors: BIANCO, James, A.; 10453 Maplewood Place S.W., Seattle, WA 98146 (US). BURSTEN, Stuart, L.; 36116 S.E. 89th Place, Snoqualmie, WA 98065 (US).

(74) Agent: OSTER, Jeffrey, B.; Cell Therapeutics, Inc., Suite 400, 201 Elliott Avenue West, Seattle, WA 98119 (US).

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(57) Abstract

There is disclosed a compound, pharmaceutical composition and methods for increasing mesenchymal cell migration including smooth muscle cells and endothelial cells and for peripheral nerve regeneration. This will promote tensile strength and rapid vascularization of wounds while accelerating the healing process. The inventive pharmaceutical compositions act as a platelet derived growth factor (PDGF), fibroblast derived growth factor (FGF), epidermal derived growth factor (EGF), vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) agonist, comprising an effective amount of a phosphatidic acid (PA) or bis PA species, or combination thereof. The pharmaceutical composition is useful for the treatment of wound healing or surgical healing of skin, vasculature, soft tissue or bone due to physical trauma, surgical procedures, fractures of bone or spinal cord, burns or soft tissue injury. The pharmaceutical composition is also useful for the treatment of tissue regeneration in areas of devascularized tissue or organ trauma, including central nervous system tissue and peripheral nervous tissue.

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COMPOSITION FOR WOUND HEALING, NEURON GROWTH AND VASCULARIZATION

Technical Field of the Invention

The present invention provides a compound, pharmaceutical composition and methods for increasing mesenchymal cell migration including smooth muscle cells and endothelial cells and for peripheral nerve regeneration. This will promote tensile strength and rapid vascularization of wounds while accelerating the healing process. The inventive pharmaceutical compositions act as a platelet derived growth factor (PDGF), fibroblast derived growth factor (FGF), epidermal derived growth factor (EGF), vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) agonist, comprising an effective amount of a phosphatidic acid (PA) or bis PA species, or combination thereof. The pharmaceutical composition is useful for the treatment of wound healing or surgical healing of skin, vasculature, soft tissue or bone due to physical trauma, surgical procedures, fractures of bone or spinal cord, burns or soft tissue injury. The pharmaceutical composition is also useful for the treatment of tissue regeneration in areas of devascularized tissue or organ trauma, including central nervous system tissue and peripheral nervous tissue, vascularization of the myocardium after an infarction.

Background of the Invention

Angiogenesis is a fundamental process by which new blood vessels are formed. It is essential in reproduction, development and wound repair. Wound repair includes, for example, healing of peptic ulcers, myocardial infarctions and third-degree burns as dependent upon angiogenesis. In searching for angiogenic agonists, only polypeptide molecules have been identified. Such molecules most likely exert their activity outside of the cell. No intracellular-acting angiogenic small molecules have been identified. Angiogenic molecules include, for example, fibroblast growth factors (FGF), platelet derived growth factor (PDGF), transforming growth factor alpha and beta (TGF-α and TGF-β), angiogenin, tumor necrosis factor alpha (TNF-α), angiotropin, and vascular endothelial growth factor (VEGF).

Both FGF and VEGF are potent angiogenic factors which induce formation of new capillary blood vessels. VEGF is an endothelial cell-specific mitogen and an angiogenesis inducer that is released by a variety of tumor cells and expressed in human tumor cells *in situ*. Unlike FGF, transfection of cell lines with a cDNA sequence encoding VEGF did not promote transformation, but did facilitate tumor growth *in vivo* (Ferrara et al., *J. Clin. Invest.* 91:160, 1993). This was likely due to paracrine stimulation of neovasculogenesis.

In surgical fields, a technique to augment healing of complex bone fractures, such as spinal cord injury, has developed that involves omentum transplantation. These techniques have evolved in view of reports published in China and in the *Central African Journal of*

Medicine to increase healing of tissue, including bone tissue and CNS tissue, when having omentum in intimate contact with the injured site.

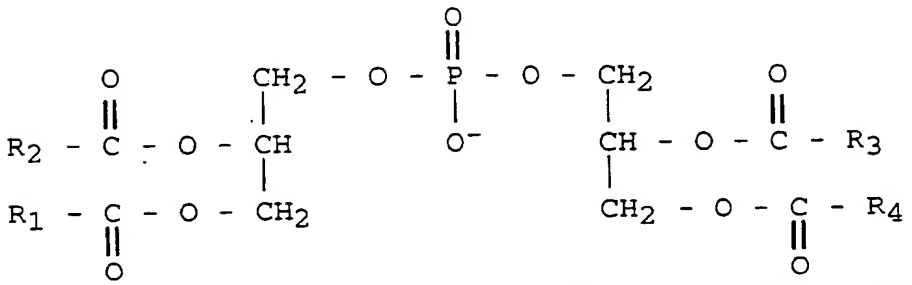
Moreover, there is a need in the art for a pharmaceutical composition to aid in the healing of neuronal tissue due to trauma or stroke due to devascularization and for peripheral nervous tissue.

Therefore, there is a need in the art to develop small molecule agonists of PDGF, FGF, EGF, VEGF, and NGF individually or as a group. Moreover, if these cytokines signal through a common second messenger pathway within the cell, such agonists will have broad therapeutic activity useful for the treatment of wound healing or surgical healing of skin, vasculature, soft tissue or bone due to physical trauma, surgical procedures, fractures of bone or spinal cord, burns or soft tissue injury. The present invention was made by discovering a common signaling mechanism, and discovering a common intracellular signaling intermediate.

Summary of the Invention

The present invention provides a compound, pharmaceutical composition and methods for increasing mesenchymal cell migration including smooth muscle cells and endothelial cells and for neuronal cell regeneration and embryogenesis. This will promote tensile strength and rapid vascularization of wounds while accelerating the healing process. For neuronal tissue, the inventive compound and pharmaceutical composition will promote neuronal cell regeneration due to areas of devascularized tissue or traumatic injury *(e.g.*, reinnervation of CNS or peripheral nerves such as the spinal chord) and mimic activity of brain-derived growth factors such as CNTF (ciliary neurotrophic factor). The inventive pharmaceutical compositions act as a platelet derived growth factor (PDGF), CNTF, fibroblast derived growth factor (FGF), epidermal derived growth factor (EGF) and vascular endothelial growth factor (VEGF) agonist, comprising an effective amount of a phosphatidic acid (PA) species, or combination thereof. The present invention provides a method for treating wounds or surgical healing of skin, vasculature, soft tissue or bone due to physical trauma, surgical procedures, fractures of bone or spinal cord, burns or soft tissue injury, comprising administering an effective amount of a non-arachidonoyl phosphatidic acid (PA) selected from the group consisting of Group I PA's, Group II PA's, Group IIa PA's, Group III PA's, Group IV PA's, bis PA's, hemi (bis) PA's, N-acylethanolamines, and combinations thereof. Group I PA's comprise LPAAT-derived (lysophosphatidic acyl transferase) PA's with a >90% C_1-C_{18} saturated and unsaturated and a >95% linoleate component in the sn-2 position. Group II PA's comprise LPAAT-derived PA's with an alkyl or alkenyl (at least 90% C_{18}) in the sn-1 position and >80% linoleate in the sn-2 position. Group IIa PA's comprise alkyl myristate or acyl myristate in the sn-1 position and a C_{18} unsaturated (acyl) hydrocarbon in the sn-2 position. Group III PA's comprise an sn-1 alkyl C_{18} hydrocarbon and any hydrocarbon in the sn-2 position. Group IV PA's comprise an oleoyl ($C_{18:1}$:ω-9) in

the sn-2 position and are specifically derived from PC (phosphatidyl choline) PLD (phospholipase D). Bis PA's comprise bis (diacylglycero) (phosphate) having four acyl chains (R_1-R_4) according to the following formula I:

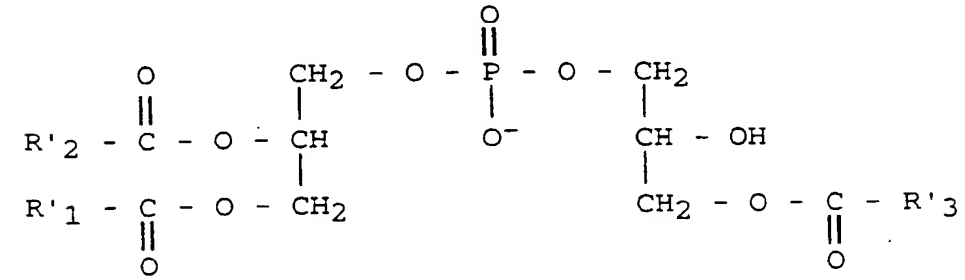


wherein the following molecular weight species have the noted acyl chains according to Table 1:

Table 1

Mol. Wt.	R_1	R_2	R_3	R_4
1387	(20:4)	(20:4)	(20:4)	(20:4)
1420	(18:1) oleate or	(22:2) or	(22:2) or	(18:1) oleate or
1422	(18:0) stearate	(22:3)	(22:3)	(18:0) stearate
1437	(o-18:0) o-octadecayl	(20:4)	(o-24:0)	(22:1)
1437	(o-18:0) o-octadecayl	(20:4)	(22:1)	(o-24:0)
1488	(18:0) stearate	(22:2)	(22:1)	(o-24:0)
1488	(18:0) stearate	(22:3)	(22:1)	(o-24:0)

Additionally, bis PA's comprise hemi-bis (diacylglycero) (phosphate) having three acyl chains (R'_1-R'_3) according to the following formula II:



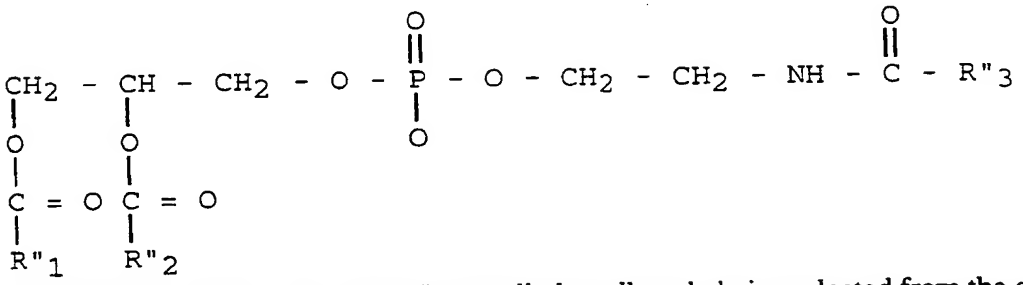
wherein the following molecular weight species have the noted acyl chains according to Table 2:

Table 2

Mol. Wt.	R'_1	R'_2	R'_3
1011	(18:1)	(18:2)	(o-16:0)
1024	(o-18:1)	(18:2)	(o-18:1)
1024	(18:1)	(18:2)	(16:1)

1034	(o-18:2)	(18:2)	(18:2)
1081	(18:0)	(20:4)	(16:0)

Additionally, N-acylethanolamines having three acyl chains ($R"_1$-$R"_3$) according to the following formula III:



wherein each of $R"_1$, $R"_2$ and $R"_3$ are alkyl or alkenyl chains selected from the group consisting of 18:0, 18:1, 18:2, 18:3 and 20:4. Preferably, $R"_1$ is 18:0, 18:1 or 18:2; $R"_2$ is 18:2, 18:3 or 20:4; and $R"_3$ is 18:2 or 20:4.

The following PA's, including, for example, 1-o-octadecanyl 2-oleoyl PA (687), 1-oleoyl 2-linoleoyl PA (697 or 698), 1-o-octadecanyl 2-linoleoyl PA (681), 1-o-'en-octadecanyl-9,12-dienyl 2-linoleoyl PA (679), 1-myristoyl 2-oleoyl PA (645), 1-o-myristoyl 2-stearoyl PA (633), 1,2-sn-dilinoleoyl PA (695), 1-stearoyl 2-oleoyl PA (701), 1-o-oleoyl 2-20:4 PA (707), 1-o-linoleoyl 2-20:4 PA (705), 1-o-linoleoyl 2-20:5 PA (703), and combinations thereof comprise the inventive pharmaceutical composition. The numbers in parenthesis next to each PA species show the approximate molecular weight of the PA species as seen by mass spectroscopy analysis. The present invention further provides a method for promoting tissue regeneration in areas of devascularized tissue and for promoting embryogenesis *ex vivo* by promoting cellular growth and differentiation, comprising administering an effective amount of a PAs selected from the group consisting of Group I PA's, Group II PA's, Group IIa PA's, Group III PA's, Group IV PA's, bis PA's and combinations thereof. The inventive method for tissue regeneration in areas of devascularized tissue further comprises healing of neuronal tissue and reineravation of CNS and peripheral nerves due to devascularization or trauma or stroke.

The present invention further provides a pharmaceutical composition for local administration to a wound site or a site in or on a patient requiring angiogenic activity, comprising aphosphatidic acid (PA) selected from the group consisting of Group I PA's, Group II PA's, Group IIa PA's, Group III PA's, Group IV PA's, bis PA's and combinations thereof and a pharmaceutically acceptable excipient.

Brief Description of the Drawings

Figure 1 illustrates the stimulation of proliferation of Balb/3T3 cells, a murine fibroblast cell line, by L-α-Phosphatidic acid. Cellular proliferation was determined by tritiated thymidine incorporation. The PAs were either synthesized or purchased (Avanti Polar-Lipids, Inc. Alabaster AL), dissolved in chloroform, dried under N_2 and stored under

argon. The PAs were reconstituted in phosphate buffered saline containing 0.1% fatty acid-free bovine serum albumin by sonication on ice. The concentrations of PAs are shown in the X axis.

Figure 2 illustrates the stimulation of proliferation of Balb/3T3 cells by dilauroyl PA (PA2). Cellular proliferation was determined by tritiated thymidine incorporation. The PAs were either synthesized or purchased (Avanti Polar-Lipids, Inc. Alabaster AL), dissolved in chloroform, dried under N_2 and stored under argon. The PAs were reconstituted in phosphate buffered saline containing 0.1% fatty acid-free bovine serum albumin by sonication on ice. The concentrations of PAs are shown in the X axis.

Figure 3 illustrates the stimulation of proliferation of Balb/3T3 cells by Dioleoyl PA. Cellular proliferation was determined by tritiated thymidine incorporation. The PAs were either synthesized or purchased (Avanti Polar-Lipids, Inc. Alabaster AL), dissolved in chloroform, dried under N_2 and stored under argon. The PAs were reconstituted in phosphate buffered saline containing 0.1% fatty acid-free bovine serum albumin by sonication on ice. The concentrations of PAs are shown in the X axis.

Figure 4 illustrates the stimulation of proliferation of Balb/3T3 cells by 1-alkyl-oleoyl 2-oleoyl PA. Cellular proliferation was determined by tritiated thymidine incorporation. The PAs were either synthesized or purchased (Avanti Polar-Lipids, Inc. Alabaster AL), dissolved in chloroform, dried under N_2 and stored under argon. The PAs were reconstituted in phosphate buffered saline containing 0.1% fatty acid-free bovine serum albumin by sonication on ice. The concentrations of PAs are shown in the X axis.

Figures 5-7 illustrate a mass spectrograph of a designated lipid fraction isolated from Balb 3T3 fibroblasts stimulated with PDGF (25 ng/ml). Specifically, Figure 5 shows a mass spec of a PA HPLC peak 5 seconds after stimulation with PDGF including the 1-o-alkyl C18 PA derivatives including 679 (1-o-'en-octadeca-9,12-dienyl 2 linoleoyl PA), 681 (1-o-octadeca-9,12-dienyl 2-linoleoyl PA), 683 (1-o-octadeca-9-enyl 2-linoleoyl PA), and related PA derivatives with C_{20} sn-2 components such as 703 (1-o-'en-octadeca-9,12-dienyl 2-arachidonoyl PA), and 707 (1-o-octadeca-9-enyl 2-arachidonoyl PA). Figure 6 shows that synthesis of Group II PA species (especially 679 and 681) was maintained after 15 seconds of stimulation with PDGF. Group II PA species include, for example, 1-o-octadecanyl 2-oleoyl PA (687), 1-oleoyl 2-linoleoyl PA (697 or 698), 1-o-octa 9,12-decadiennyl 2-linoleoyl PA (681), 1-o-'en-octadecanyl-9,12-dienyl 2-linoleoyl PA (679), 1-myristoyl 2-oleoyl PA (645), and 1-o-myristoyl 2-stearoyl PA (633) PA species. Figure 7 further shows the 15 second stimulation maintaining the Group II PA species and, in addition, 673 (1-palmitoyl, 2-oleoyl PA) and 671 (1-palmitoyl, 2-linoleoyl PA) PA species.

Figure 8 is a four-part figure showing mass spec plots from thin layer chromatographed lipids from neutrophils. Briefly, neutrophils were stimulated with GM-CSF in order to stimulate PC-directed phospholipase D (PLD), in the presence of excess

diacylglycerol (DG) in order to generate bis(PA). Neutrophils were stimulated in the presence or absence of CT-3501, a PA signaling antagonist. The incubation with GM-CSF was stopped by addition of ice cold MeOH, the lipids were extracted and separated on thin layer chromatography (TLC). Panel A illustrates the bis (PA) that was made (due to ^{3}H-DG to follow the appropriate TLC fraction). In the presence of CT-3501 (panel B), bis (PA) was suppressed and hemi (lyso) bis PA was produced. In panel C, the species of PA produced by GM-CSF stimulation of neutrophils are seen. Such species are suppressed by CT-3501 (panel D).

Detailed Description of the Invention

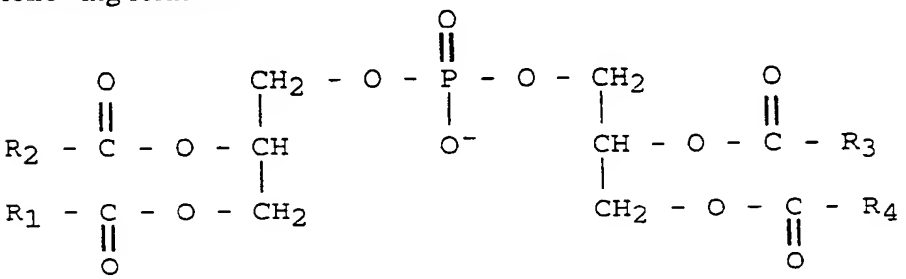
The present invention is based upon the pioneering discovery that certain PA species are produced as intracellular signaling molecules in response to pro-inflammatory stimuli mediated by, by example, PDGF, EGF, FGF and VEGF. A number of intracellular signaling events take place following PDGF, EGF, FGF or VEGF binding to their respective cell surface receptors. All of the receptors in this class of cytokines possess intrinsic tyrosine phosphorylation activity. Shortly after binding, the receptors are phosphorylated at various sites in their intracellular domain by intrinsic tyrosine kinase activity of the receptor. This leads to the creation of additional binding sites for intracellular proteins. For instance, for PDGF these include phospholipase C-γ-1 (PLC-γ-1), the *ras* GTPase activating protein (GAP), phosphatidylinositol 3 kinase (PI3kinase), pp60c-*src*, p62c-*yes*, p50-*fyn*, *Nck*, and CRB2 as well as a 120 kd and a 64 kd species. Some of the proteins that associate with the receptor are signal transduction enzymes. For example, PLC-γ-1 is a specific phosphodiesterase that produces diacylgycerol (DAG) and inositol triphosphate, two second messengers that activate a serine/threonine specific protein kinase protein kinase C (PKC) and increase intracellular calcium levels. PI3kinase is a lipid kinase that phosphorylates the D3 position of phosphatidylinositol phosphatidylinositol-4-phosphate, or PI 4,5,P2. The physiological significance of this intracellular lipid species is unclear, but mutant PDGF receptors that no longer bind PI3kinase by virtue of a substitution of a specific tyrosine residue no longer proliferate in response to PDGF. In addition, PDGF induces activation of the serine/threonine kinase MAP kinase, via MAP kinase kinase, which may be activated by activation of *ras/raf* pathway. MAP kinase acts to activate the nuclear transcription factors c-*jun*, *c-fos* and possibly c-*myc*. PDGF also up regulates increased transcription of these transcription factors.

We have discovered that PDGF, FGF, VEGF, EGF, Her2,3,4/regulin, NGF, and IGF-1 or 2 induce increased levels of a specific species group of phosphatidic acid (PA). PA can be produced by either a membrane associated lysophosphatidic acyl transferase (LPAAT), by phospholipase D catalyzed hydrolysis of phosphatidyl choline or phosphatidyl-ethanolamine, or via DAG kinase conversion of diacyl glycerol (DAG) to PA. PA is a potent

intracellular signaling lipid and can be converted to DAG by phosphatidyl phosphohydrolase (PAPH).

As shown in Figure 1-4, addition of various types of PA's , including L-a PA (derived from natural sources), 1,2 dilauroyl-sn-glycero-3-phosphate, 1,2 dioleoyl-sn-glycero-3-phosphate, 1-stearolyl-2-aracidonyl-sn-glycero-3-phosphate and 1-alkyl-oleoyl-2-oleoyl-PA, are all mitogenic in Balb/3T3 cells, a fibroblast cell line that is predictive of would healing, including vascularization and improved tensile strength.

The present invention provides a compound, pharmaceutical composition and methods for increasing mesenchymal cell migration including smooth muscle cells and endothelial cells. This will promote tensile strength and rapid vascularization of wounds while accelerating the healing process. The inventive pharmaceutical compositions act as a platelet derived growth factor (PDGF), fibroblast derived growth factor (FGF), epidermal derived growth factor (EGF) and vascular endothelial growth factor (VEGF) agonist, comprising an effective amount of a phosphatidic acid (PA) species, or combination thereof. The present invention provides a method for treating wounds or surgical healing of skin, vasculature, soft tissue or bone due to physical trauma, surgical procedures, fractures of bone or spinal cord, burns or soft tissue injury, comprising, comprising administering an effective amount of a phosphatidic acid (PA) selected from the group consisting of Group I PA's, Group II PA's, Group IIa PA's, Group III PA's, Group IV PA's, bis PA's, hemi (bis) PA's, N-acylethanolamines, and combinations thereof. Group I PA's comprise LPAAT-derived (lysophosphatidic acyl transferase) PA's with a >90% C_1-C_{18} saturated and unsaturated and a >95% linoleate component in the sn-2 position. Group II PA's comprise LPAAT-derived PA's with an alkyl or alkenyl (at least 90% C_{18}) in the sn-1 position and >80% linoleate in the sn-2 position. Group IIa PA's comprise alkyl myristate or acyl myristate in the sn-1 position and a C_{18} unsaturated (acyl) hydrocarbon in the sn-2 position. Group III PA's comprise an sn-1 alkyl C_{18} hydrocarbon and any hydrocarbon in the sn-2 position. Group IV PA's comprise an oleoyl ($C_{18:1}$:ω-9) in the sn-2 position and are specifically derived from PC (phosphatidyl choline) PLD (phospholipase D). Bis PA's comprise bis (diacylglycero) (phosphate) having four acyl chains (R_1-R_4) according to the following formula I:

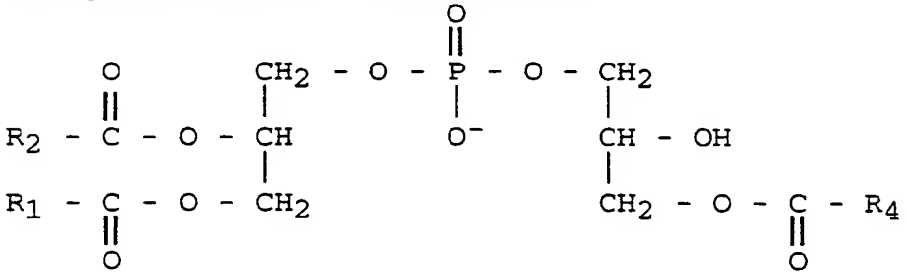


wherein the following molecular weight species have the noted acyl chains according to Table 1:

Table 1

Mol. Wt.	R_1	R_2	R_3	R_4
1387	(20:4)	(20:4)	(20:4)	(20:4)
1420	(18:1) oleate or	(22:2) or	(22:2) or	(18:1) oleate or
1422	(18:0) stearate	(22:3)	(22:3)	(18:0) stearate
1437	(o-18:0) o-octadecayl	(20:4)	(o-24:0)	(22:1)
1437	(o-18:0) o-octadecayl	(20:4)	(22:1)	(o-24:0)
1488	(18:0) stearate	(22:2)	(22:1)	(o-24:0)
1488	(18:0) stearate	(22:3)	(22:1)	(o-24:0)

Additionally, bis PA's comprise hemi-bis (diacylglycero) (phosphate) having three acyl chains (R_1-R_3) according to the following formula II:

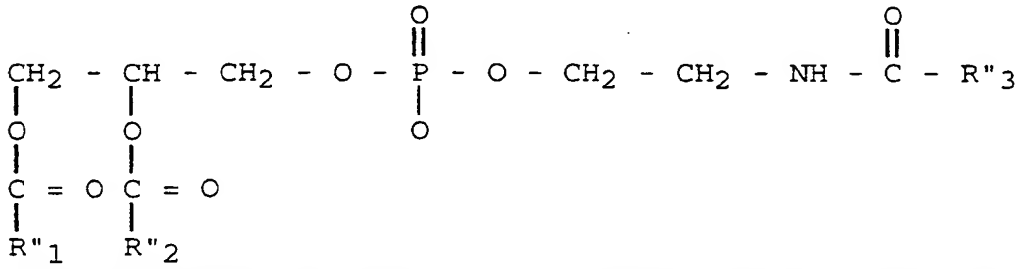


wherein the following molecular weight species have the noted acyl chains according to Table 2:

Table 2

Mol. Wt.	R_1	R_2	R_3
1011	(18:1)	(18:2)	(o-16:0)
1024	(o-18:1)	(18:2)	(o-18:1)
1024	(18:1)	(18:2)	(16:1)
1034	(o-18:2)	(18:2)	(18:2)
1081	(18:0)	(20:4)	(16:0)

Additionally, N-acylethanolamines having three acyl chains ($R"_1$-$R"_3$) according to the following formula III:



wherein each of $R"_1$, $R"_2$ and $R"_3$ are alkyl or alkenyl chains selected from the group consisting of 18:0, 18:1, 18:2, 18:3 and 20:4. Preferably, $R"_1$ is 18:0, 18:1 or 18:2; $R"_2$ is 18:2, 18:3 or 20:4; and $R"_3$ is 18:2 or 20:4.

The following PA's, including, for example, 1-o-octadecanyl 2-oleoyl PA (687), 1-oleoyl 2-linoleoyl PA (697 or 698), 1-o-octadecanyl 2-linoleoyl PA (681), 1-o-'en-octadecanyl-9,12-dienyl 2-linoleoyl PA (679), 1-myristoyl 2-oleoyl PA (645), 1-o-myristoyl 2-stearoyl PA (633), 1,2-sn-dilinoleoyl PA (695), 1-stearoyl 2-oleoyl PA (701), 1-o-oleoyl 2-20:4 PA (707), 1-o-linoleoyl 2-20:4 PA (705), 1-o-linoleoyl 2-20:5 PA (703), and combinations thereof comprise the inventive pharmaceutical composition. The numbers in parenthesis next to each PA species show the approximate molecular weight of the PA species as seen by mass spectroscopy analysis.

The present invention further provides a pharmaceutical composition for local administration to a wound site or a site in or on a patient requiring angiogenic activity, comprising a phosphatidic acid (PA) selected from the group consisting of Group I PA's, Group II PA's, Group IIa PA's, Group III PA's, Group IV PA's, bis PA's, hemi (bis) PA's, N-acylethanolamines, and combinations thereof and a pharmaceutically acceptable excipient.

Activation of phospholipase D (PLD) does not always produce PA but may also produce either bis (diacylglycerol) phosphate (Bis[PA]), bis (monoacylglycerol) phosphate (lyso(bis) PA), or closely-related species derived from phosphatidylglycerol. This was observed for a variety of glycerol forms containing free hydroxyl moieties (Tettenbron et al., *Biochem. Biophys. Res. Comm.* 155:249, 1988; and Guillemain et al., *Amer. J. Physiol.* 266:C692, 1994). Indeed, lipid metabolism focusing upon PA and its relatives as a signaling molecule and therapeutic agonist is complex and structure-dependent, particularly with regards to the makeup of its acyl chains. Accordingly, the present invention was made with the investigation of PA structure determination by HPLC and mass spectroscopy using signaling inhibitors (antagonists) as tools to identify the structure of the agonist agents. The inventive pharmaceutical compositions are signaling agonists that work by acting as intracellular messengers to up-regulate cellular activity and have a utility as therapeutic agents.

Formulation and Dosage

It will be recognized by one of skill in the art that the form and character of the pharmaceutically acceptable carrier or diluent is dictated by the amount of active ingredient *(i.e.*, PA, bis PA, hemi (bis PA, or acylethanolamine) with which it is to be combined, the route of administration and other well-known variables. A PA, bis PA, hemi (bis) PA, or acylethanolamine compound or a pharmaceutically acceptable salt or hydrate or solvate thereof is administered to a patient in an amount sufficient to increase mesenchymal cell migration. The route of administration of the compound or composition is not critical but is usually local, topical, sustained release, impregnated in a suture, sponge or wound covering, oral or parenteral. The term parenteral, as used herein, includes intravenous, intramuscular, subcutaneous, intranasal, intrarectal, transdermal, opthalmic, intravaginal or intraperitoneal administration. The subcutaneous and intramuscular forms of parenteral administration are

generally preferred. The daily parenteral dosage regimen will preferably be from about 0.01 mg/kg to about 25 mg/kg of total body weight, most preferably from about 0.1 mg/kg to about 4 mg/kg. Preferably, each parenteral dosage unit will contain the active ingredient in an amount of from about 0.1 mg to about 400 mg.

Preferably, the pharmaceutical composition is formulated for sustained release and interdispursed into an injectable matrix, such as collagen or alginate (or other complex polysaccharide) and injected or locally applied (*e.g.*, during a surgical procedure) to a site in need of angiogenic wound healing. For example, the pharmaceutical composition may be administered in the form of a skin patch, a surgical sponge or in a polymeric matrix for local organ application to an organ in need of angiogenic wound healing. Preferably, the pharmaceutical composition is impregnated in surgical sutures to augment surgical wound healing.

The pharmaceutical compositions are generally active when given orally and can be formulated as liquids, for example, syrups, suspensions or emulsions, tablets, capsules and lozenges. A liquid formulation will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s), for example, ethanol, glycerine, non-aqueous solvent, for example polyethylene glycol, oils, or water with a suspending agent, preservative, flavoring or coloring agent. A composition in the form of a tablet can be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose. A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, pellets containing the active ingredient can be prepared using standard carriers and then filled into a hard gelatin capsule. Alternatively, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), for example, aqueous gums, celluloses, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule. The daily oral dosage regimen will preferably be from about 0.01 mg/kg to about 40 mg/kg of total body weight. Preferably, each oral dosage unit will contain the active ingredient in an amount from about 0.1 mg to about 1000 mg.

It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of a compound or a pharmaceutically acceptable salt or hydrate or solvate thereof will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the particular patient being treated, and that such optimums can be determined by conventional techniques. It will also be appreciated by one of skill in the art that the optimal course of treatment (*i.e.*, the number of doses of a compound or a pharmaceutically acceptable salt or hydrate or solvate thereof given per day and duration of therapy) can be ascertained by those skilled in the art using conventional course of treatment determination tests.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following examples are, therefore, to be construed as merely illustrative and not a limitation of the scope of the present invention in any way.

Example 1

This example illustrates that various species of PA and lyso-phosphatidic acid induced Balb/3T3 cells to proliferate (Figures 1-4). The various species of PA are indicated on each figure. Phosphatidic Acid (PA's) derivatives were synthesized or purchased from Avanti Polar-Lipids, Inc., Alabaster, AL. PA's were dissolved in chloroform, dried under N_2 and stored under Argon. PA's were reconstituted in phosphate buffered saline containing 0.1% fatty-acid free bovine serum albumin by sonication on ice. As shown in Figures 1-4, addition of various types of PA's , including L-a PA (derived from natural sources), 1,2 dilauroyl-sn-glycero-3-phosphate, 1,2 dioleoyl-sn-glycero-3-phosphate, 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphate and 1-alkyl-oleoyl-2-oleoyl-PA, were mitogenic in Balb/3T3 cells.

Figures 5-7 illustrate a mass spectrograph of a designated lipid fraction isolated from Balb 3T3 fibroblasts stimulated with PDGF (25 ng/ml). Specifically, Figure 5 shows a mass spec of a PA HPLC peak 5 seconds after stimulation with PDGF including the 1-o-alkyl C18 PA derivatives including 679 (1-o-'en-octadeca-9,12-dienyl 2 linoleoyl PA), 681 (1-o-octadeca-9,12-dienyl 2-linoleoyl PA), 683 (1-o-octadeca-9-enyl 2-linoleoyl PA), and related PA derivatives with C20 sn-2 components such as 703 (1-o-'en-octadeca-9,12-dienyl 2-arachidonoyl PA), and 707 (1-o-octadeca-9-enyl 2-arachidonoyl PA). Figure 6 shows that synthesis of Type 1B PA species (especially 679 and 681) was maintained after 15 seconds of stimulation with PDGF. Type 1B PA species include, for example, 1-o-octadecanyl 2-oleoyl PA (687), 1-oleoyl 2-linoleoyl PA (697 or 698), 1-o-octa9,12-decadienyl 2-linoleoyl PA (681), 1-o-'en-octadecanyl-9,12-dienyl 2-linoleoyl PA (679), 1-myristoyl 2-oleoyl PA (645), and 1-o-myristoyl 2-stearoyl PA (633) PA species. Figure 7 further shows the 15 second stimulation maintaining the Type 1B PA species and, in addition, 673 (1-palmitoyl, 2-oleoyl PA) and 671 (1-palmitoyl, 2-linoleoyl PA) PA species.

Example 2

This example illustrates a synthesis procedure for 1-o-alkyl-2-acyl PA (C:18:1, C:18:1) in four steps. Initially, 1-o-oleyl glycerol (1 g) was reacted with 1.2 mols of trityl chloride. The resulting 1-o-alkyl-3 trityl glycerol was purified by column chromatography (yield 1.81 g). Trityl protected compound was reacted with oleic anhydride (1.25 mole) and the product, 1-0-alkyl-2-acyl-3-trityl, was purified by column chromatography (yield 1.637 g). The product was detritylated and again purified on a silica column (yield 500 mg) and reacted with phosphorous oxychloride to obtain the required phosphatidic acid (yield 200

mg). The PA was partially purified on silica column (yield 100 mg) and gave two spots on TLC. The final purification was achieved by HPLC (22 mg). The final characterization of the compound was achieved by FAB/MS.

Approximately 10 mg of the pure PA was subjected to phospholipase A to produce lyso-PA enzymatically and to test if any migration of oleic acid had occurred during synthesis. Characterization of the Lyso-PA was achieved by FAB/MS.

Example 3

This example illustrates a different synthesis of PA using the procedure described in example 2 except a different procedure for acylation. Instead of using commercially available oleic anhydride, oleic acid and 1,1 carbonyldiimidazol was used for acylation.

Example 4

This example illustrates a synthesis of PA (18:2,18;2) 1-o-(9-12) octadecyl-2-linoleoyl-PA in 6 steps. Commercially available 2,3 isopropylidene glycerol (Sigma) was reacted with linoleoyl methane sulfonate in the presence of sodium hydride to obtain 1-o-(9-12) octadecyl-2,3 isopropylidene glycerol. The resulting compound was deacetonated in the presence of 10% HCl to obtain 1-o-(9-12) octadecyl glycerol. The remaining steps are same as described in example 2.

Example 5

This example illustrates an experiment with lipid analysis of stimulated neutrophils stimulated with GM-CSF. Briefly, neutrophils were stimulated with GM-CSF in order to stimulate PC-directed phospholipase D (PLD), in the presence of excess diacylglycerol (DG) in order to generate bis (PA). Bis (PA) was formed from PA and DG conjugation by PLD using DG as an alcohol to transphosphatidylate PA. Neutrophils were stimulated in the presence or absence of CT-3501, a PA signaling antagonist. The incubation with GM-CSF was stopped by addition of ice cold MeOH, the lipids were extracted and separated on thin layer chromatography (TLC). Panel A illustrates the bis (PA) that was made (due to ^{3}H-DG to follow the appropriate TLC fraction). In the presence of CT-3501 (panel B), bis (PA) was suppressed and hemi (lyso) bis PA was produced. In panel C, the species of PA produced by GM-CSF stimulation of neutrophils are seen. Such species are suppressed by CT-3501 (panel D).

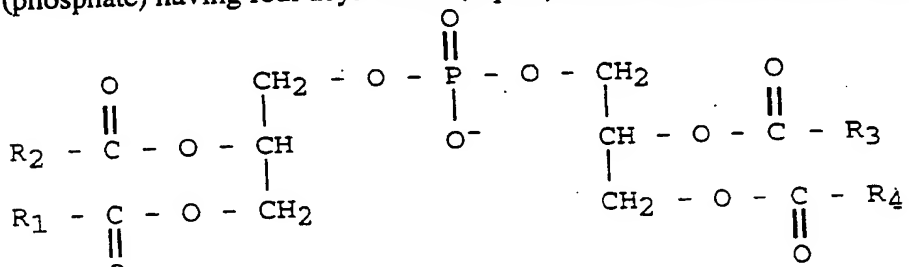
We claim:

1. A method for increasing mesenchymal cell migration to promote tensile strength and rapid vascularization of wounds, or a method for promoting tissue regeneration in areas of devascularized tissue and for promoting embryogenesis *ex vivo* by promoting cellular growth and differentiation, comprising administering an effective amount of a phosphatidic acid (PA) selected from the group consisting of Group I PA's, Group II PA's, Group IIa PA's, Group III PA's, Group IV PA's, bis PA's, hemi (bis) PA's, N-acylethanolamines, and combinations thereof.

2. The method of claim 1 wherein increasing mesenchymal cell migration allows for a treatment of wounds or surgical healing of skin, vasculature, soft tissue or bone due to physical trauma, surgical procedures, fractures of bone, peripheral nerve tissue or spinal cord, burns or soft tissue injury.

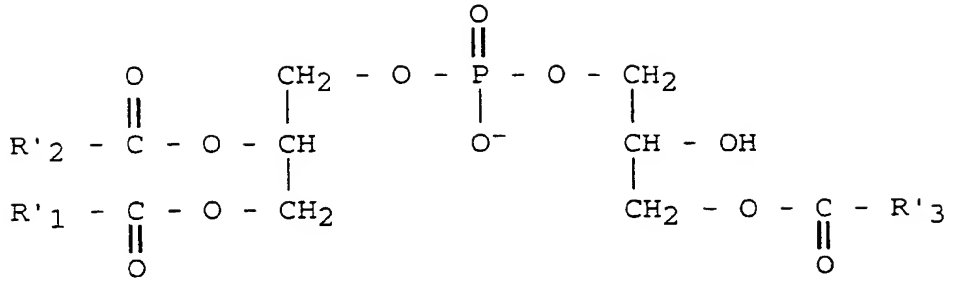
3. The method of claim 1 wherein the phosphatidic acid (PA) is selected from the group consisting of 1-o-octadecanyl 2-oleoyl PA (687), 1-oleoyl 2-linoleoyl PA (697 or 698), 1-o-octadecanyl 2-linoleoyl PA (681), 1-o-octadecanyl-9,12-dienyl 2-linoleoyl PA (679), 1-myristoyl 2-oleoyl PA (645), 1-o-myristoyl 2-stearoyl PA (633), 1,2-sn-dilinoleoyl PA (695), 1-oleoyl 2-linoleoyl PA (697), 1-stearoyl 2-oleoyl PA (701), 1-o-oleoyl 2-20:4 PA (707), 1-o-linoleoyl 2-20:4 PA (705), 1-o-linoleoyl 2-20:5 PA (703), and combinations thereof.

4. The method of claim 1 wherein the bis PA's comprise bis (diacylglycero) (phosphate) having four acyl chains (R_1-R_4) according to the following formula I:



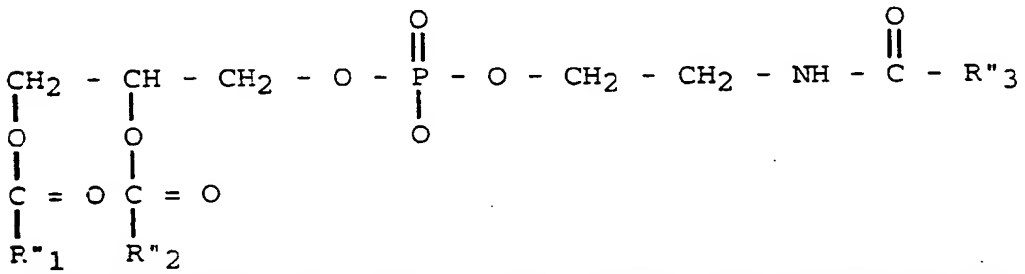
wherein R_1, R_2, R_3, and R_4 are independently selected from the group consisting of 20:4, o-20:4, 16:0, 16:1, 18:2, o-18:2, 18:1, 18:0, 22:2, 22:3, o-18:0, o-24:0, 22:1, 24:0, o-24:0, and combinations thereof.

5. The method of claim 1 wherein the hemi-bis (diacylglycero) (phosphate) PA's have having three acyl chains (R'_1-R'_3) according to the following formula II:



wherein R_1, R_2, R_3, and R_4 are independently selected from the group consisting of 20:4, o-20:4, 16:0, 16:1, 18:2, o-18:2, 18:1, 18:0, 22:2, 22:3, o-18:0, o-24:0, 22:1, 24:0, o-24:0, and combinations thereof.

6. The method of claim 1 wherein the N-acylethanolamines having three acyl chains ($R"_1$-$R"_3$) according to the following formula III:



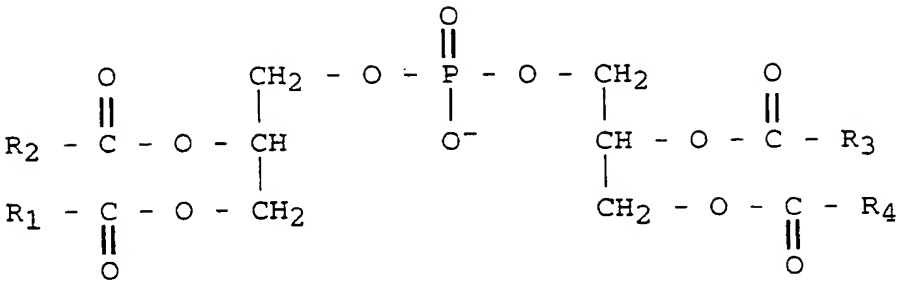
wherein each of $R"_1$, $R"_2$ and $R"_3$ are alkyl or alkenyl chains selected from the group consisting of 18:0, 18:1, 18:2, 18:3 and 20:4.

7. The method of claim 6 wherein $R"_1$ is 18:0, 18:1 or 18:2; $R"_2$ is 18:2, 18:3 or 20:4; and $R"_3$ is 18:2 or 20:4.

8. A pharmaceutical composition for local administration to a wound site or a site in or on a patient requiring angiogenic activity, comprising a phosphatidic acid (PA) selected from the group consisting of Group I PA's, Group II PA's, Group IIa PA's, Group III PA's, Group IV PA's, bis PA's, hemi (bis) PA's, N-acylethanolamines, and combinations thereof, and a pharmaceutically acceptable excipient.

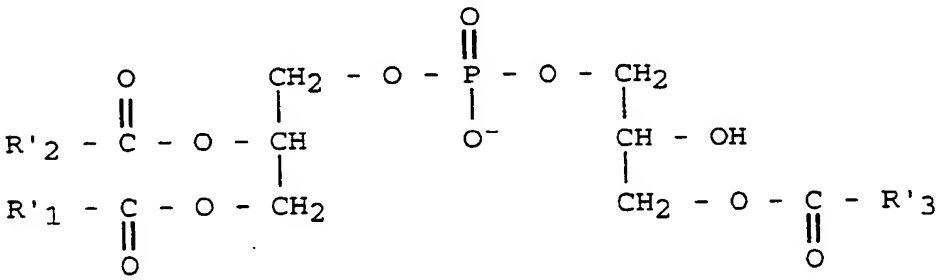
9. The pharmaceutical composition of claim 8 wherein the phosphatidic acid (PA) is selected from the group consisting of 1-o-octadecanyl 2-oleoyl PA (687), 1-oleoyl 2-linoleoyl PA (697 or 698), 1-o-octadecanyl 2-linoleoyl PA (681), 1-o-octadecanyl-9,12-dienyl 2-linoleoyl PA (679), 1-myristoyl 2-oleoyl PA (645), 1-o-myristoyl 2-stearoyl PA (633), 1,2-sn-dilinoleoyl PA (695), 1-oleoyl 2-linoleoyl PA (697), 1-stearoyl 2-oleoyl PA (701), 1-o-oleoyl 2-20:4 PA (707), 1-o-linoleoyl 2-20:4 PA (705), 1-o-linoleoyl 2-20:5 PA (703), and combinations thereof.

10. The pharmaceutical composition of claim 8 wherein the bis PA's comprise bis (diacylglycero) (phosphate) having four acyl chains (R_1-R_4) according to the following formula I:



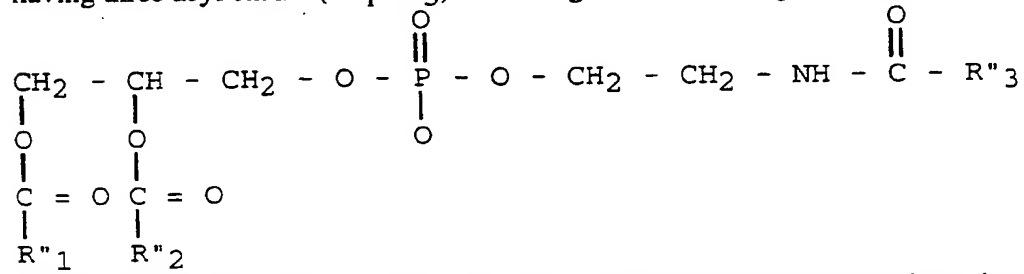
wherein R_1, R_2, R_3, and R_4 are independently selected from the group consisting of 20:4, o-20:4, 16:0, 16:1, 18:2, o-18:2, 18:1, 18:0, 22:2, 22:3, o-18:0, o-24:0, 22:1, 24:0, o-24:0, and combinations thereof.

11. The pharmaceutical composition of claim 8 wherein the hemi-bis (diacylglycero) (phosphate) PA's have having three acyl chains (R'_1-R'_3) according to the following formula II:



wherein R_1, R_2, R_3, and R_4 are independently selected from the group consisting of 20:4, o-20:4, 16:0, 16:1, 18:2, o-18:2, 18:1, 18:0, 22:2, 22:3, o-18:0, o-24:0, 22:1, 24:0, o-24:0, and combinations thereof.

12. The pharmaceutical composition of claim 8 wherein the N-acylethanolamines having three acyl chains ($R"_1$-$R"_3$) according to the following formula III:



wherein each of $R"_1$, $R"_2$ and $R"_3$ are alkyl or alkenyl chains selected from the group consisting of 18:0, 18:1, 18:2, 18:3 and 20:4.

13. The pharmaceutical composition of claim 12 wherein $R"_1$ is 18:0, 18:1 or 18:2; $R"_2$ is 18:2, 18:3 or 20:4; and $R"_3$ is 18:2 or 20:4.

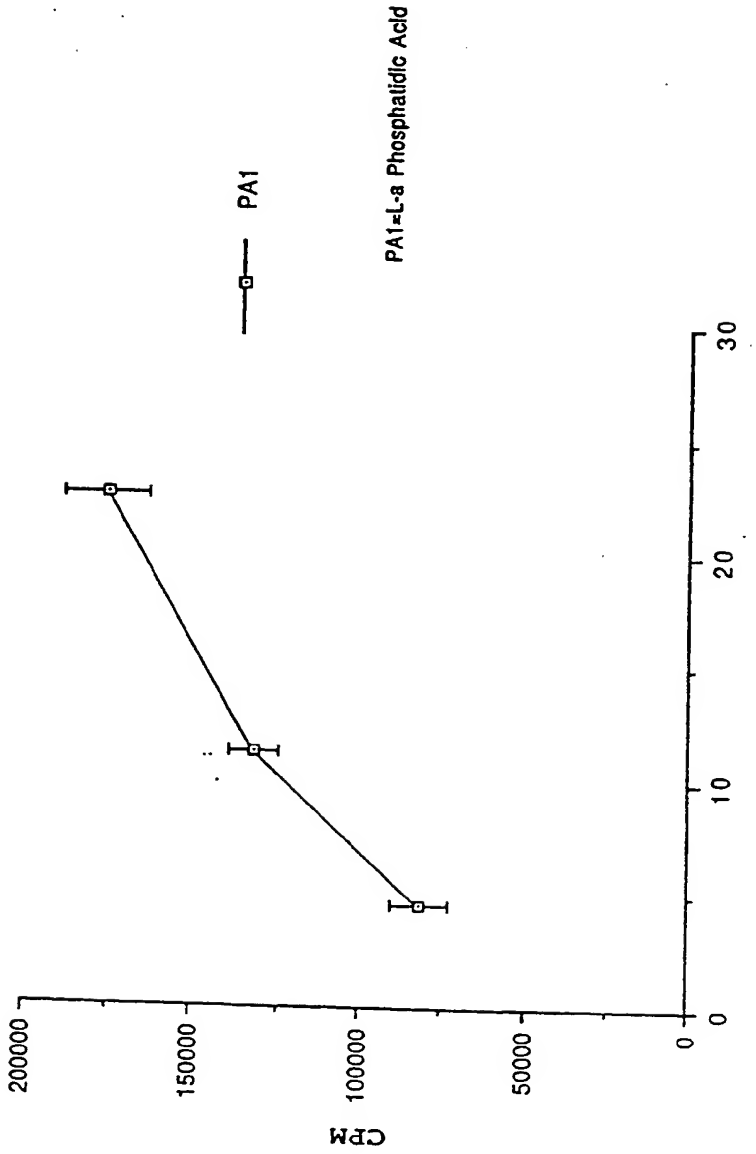


FIGURE 1

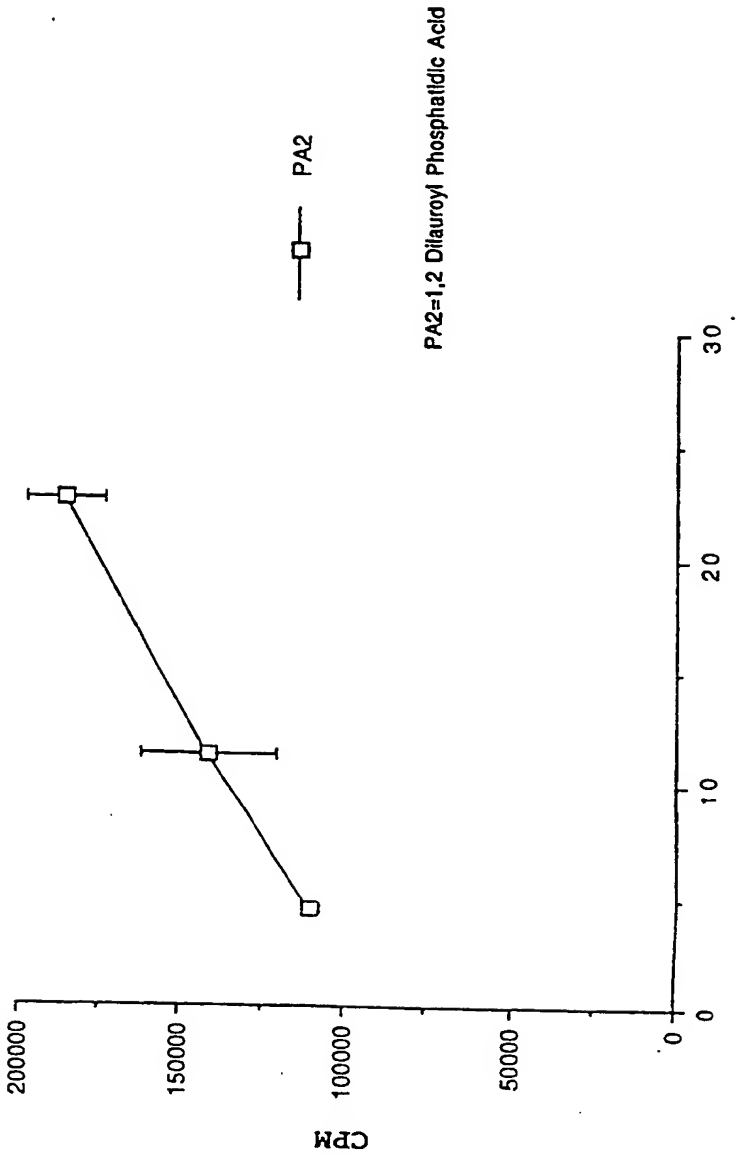


FIGURE 2

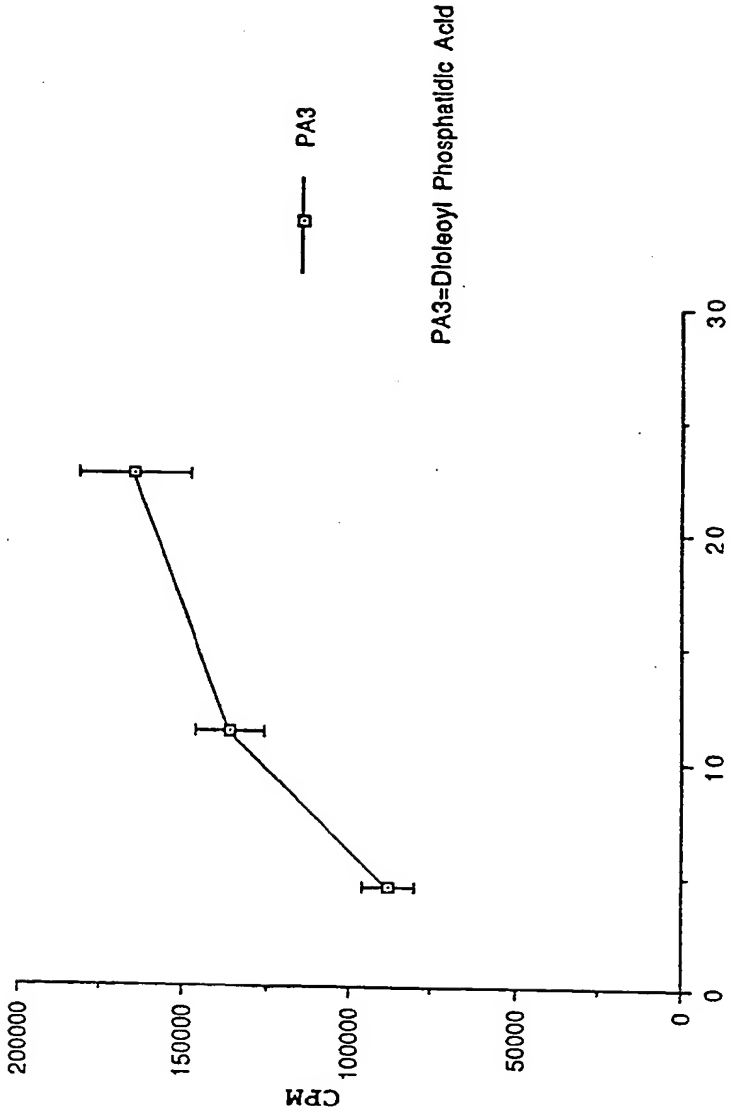
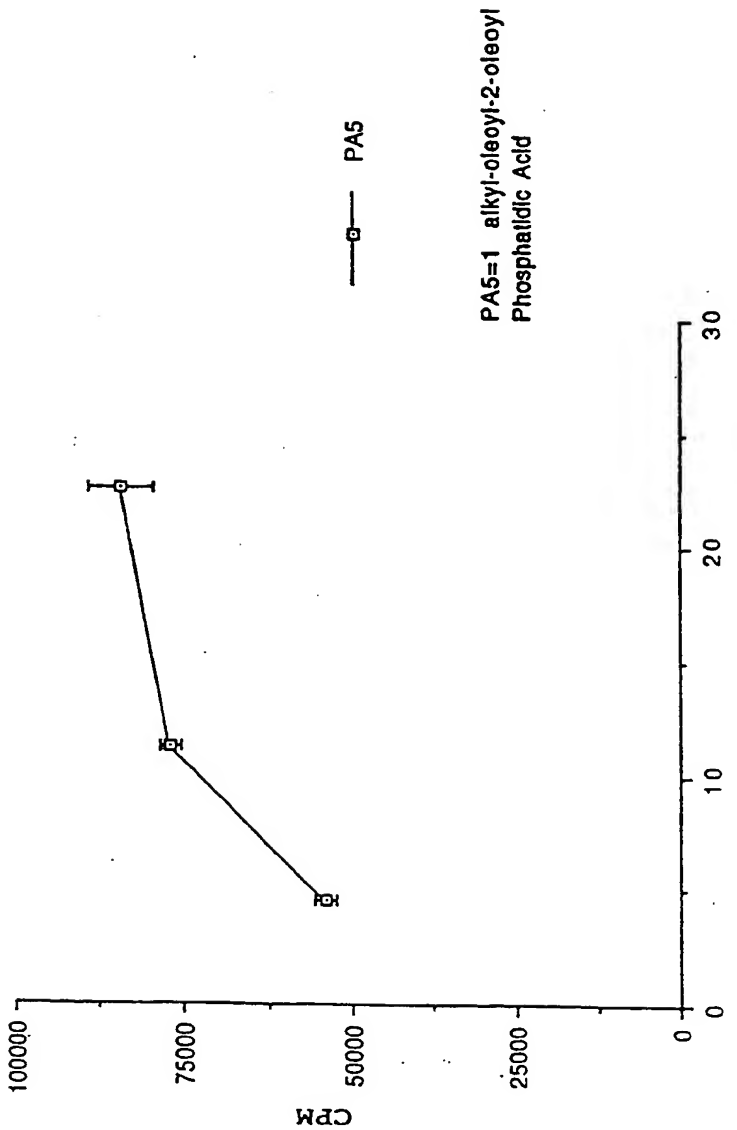


FIGURE 3



FIGUE 4

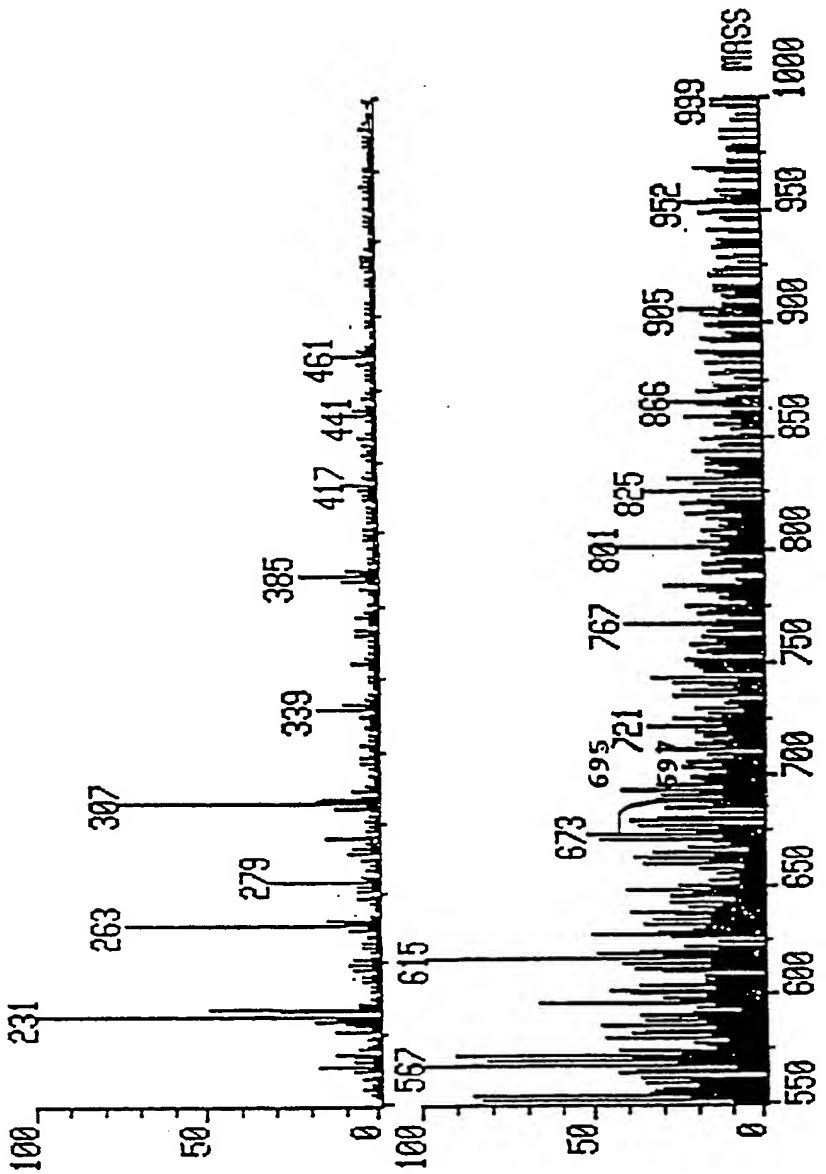


FIGURE 5

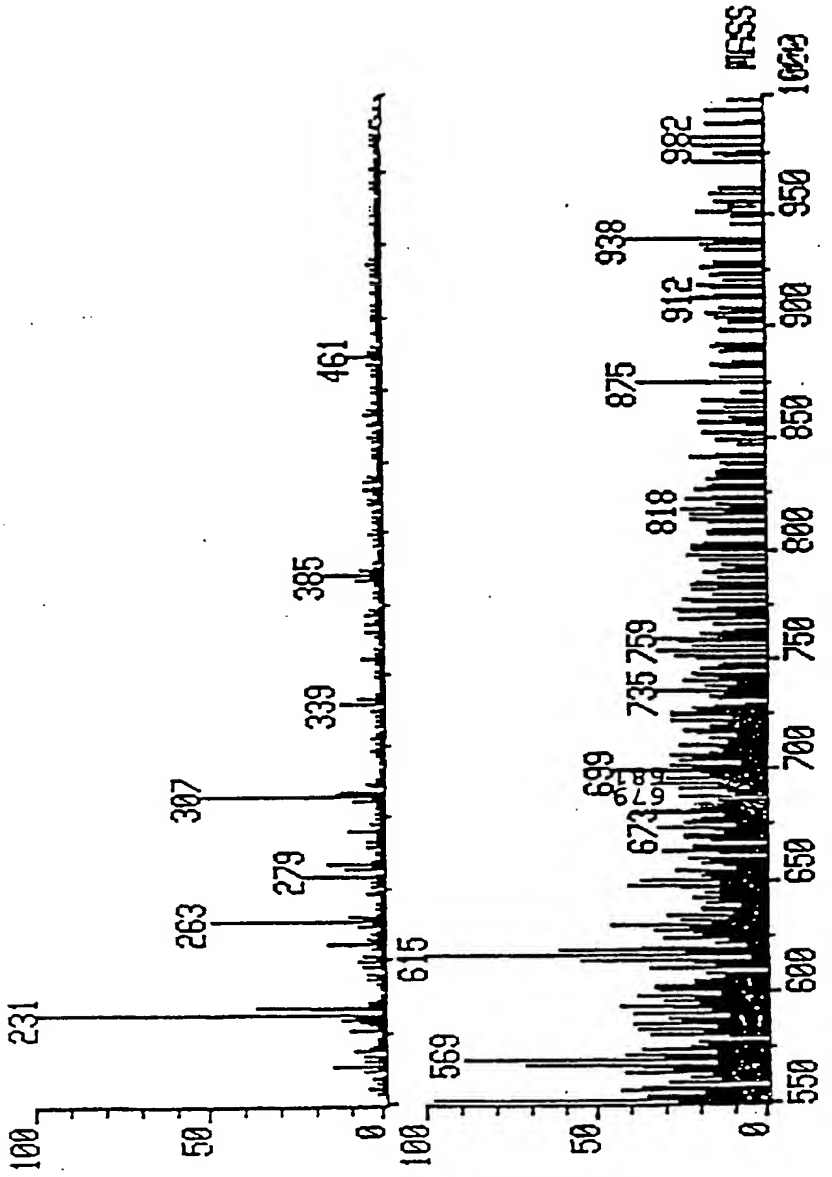


FIGURE 6

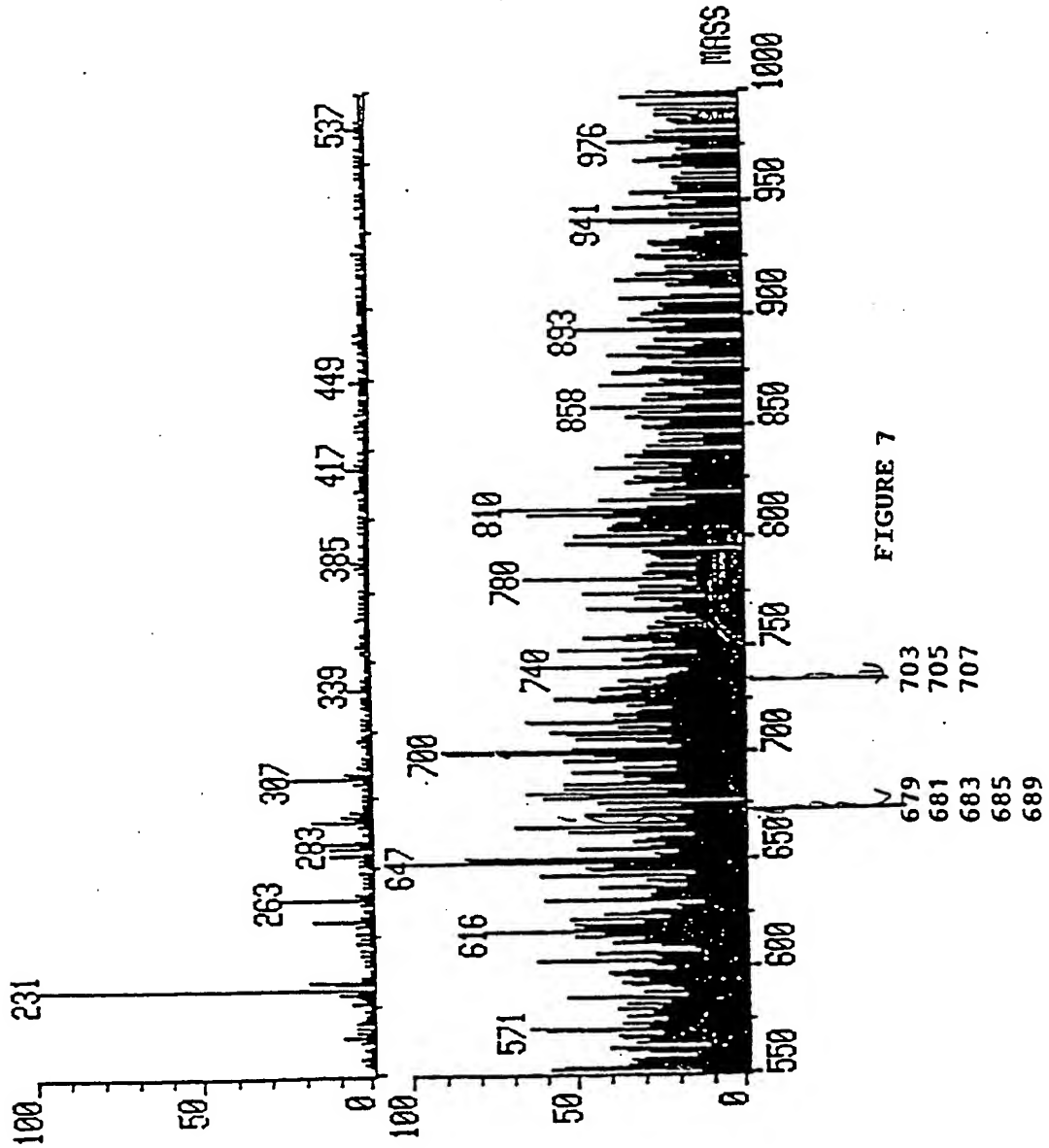


FIGURE 7

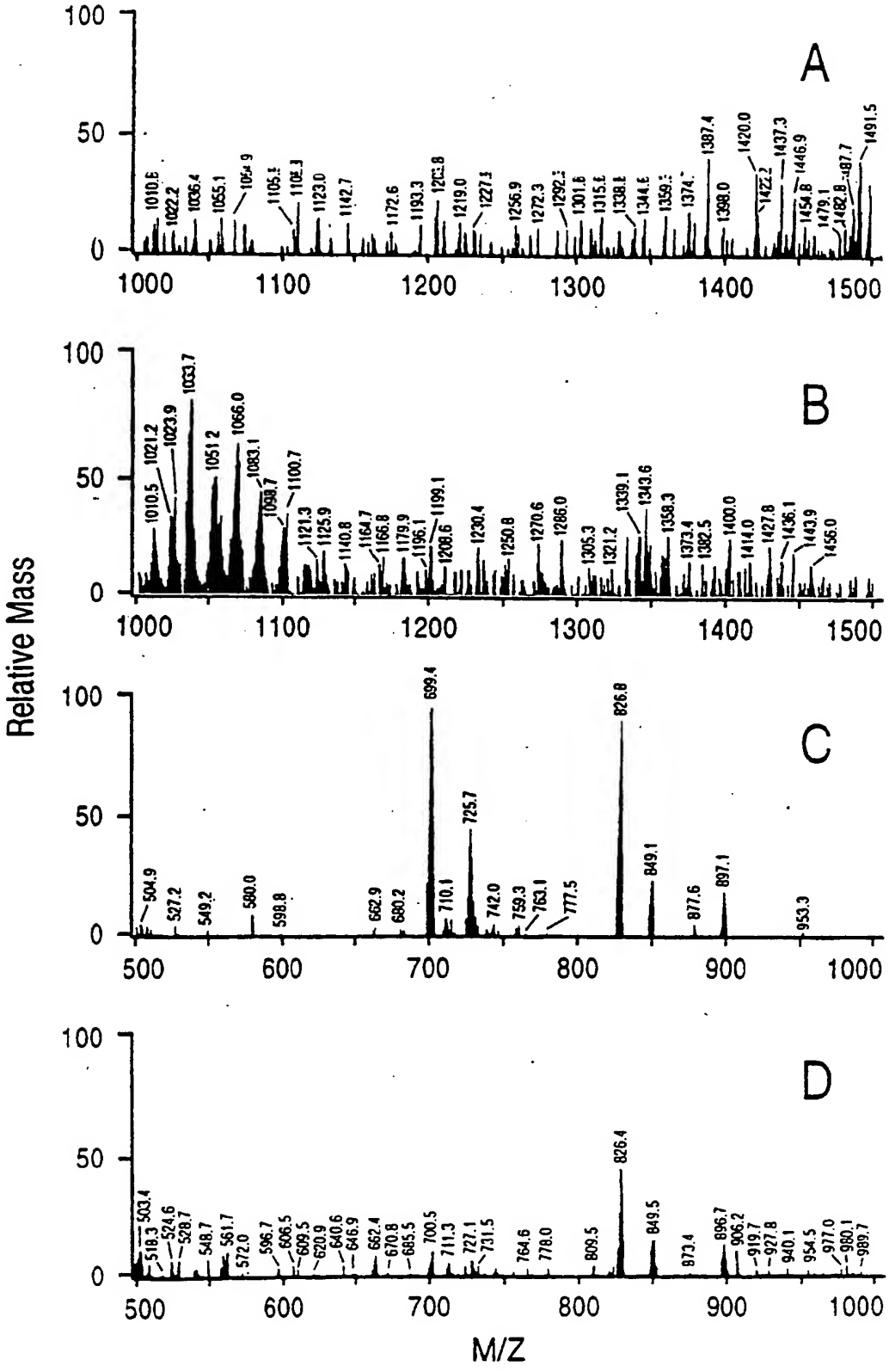


FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/01473

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/66

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE WPI Week 9421 Derwent Publications Ltd., London, GB; AN 94-173655 & JP-A-06 116 149 (EISAI CO LTD) , 26 April 1994 see abstract ---	1,2,8
P,X	EP,A,0 606 590 (RHONE-POULENC RORER GMBH) 20 July 1994 see page 2, line 16 - page 3, line 5 see page 5, line 14 - line 30 ---	8,12,13
X	DE,A,27 56 866 (A.NATTERMANN & CIE GMBH) 21 June 1979	8,12,13
Y	see claims 1,4 ---	1-7
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 June 1995	14. 07. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Tzschoppe, D

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INTERNATIONAL SEARCH REPORT

International A ation No
PCT/US 95/01473

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	see claims 1-12	1-7

Y	PROC.NATL.ACAD.SCI. USA, vol. 86, no. 11, 1989 pages 4122-4126, WALTER IMAGAWA ET AL 'Phospholipids containing polyunsaturated fatty acyl groups are mitobenic for normal mouse mammary epithelial cells in serum-free primary cell culture' see abstract	1-7

Y	J.TRAUMA, vol. 30, no. 12sup, 1990 pages S129-S133, DOUGLAS T. CROMACK ET AL. 'Current concepts in wound healing: Growth factor and macrophage interaction' see abstract	1-7

X	LIPIDS, vol. 17, no. 11, 1982 pages 798-802, QUOC QUAN DANG ET AL. 'Synthesis and identification of bis(diacylglycero)phosphoric acid and bis(monoacylglycero)phosphoric acid' see the whole document	10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/01473

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EP-A-606590	20-07-94	DE-A-	4305553	23-06-94
		CA-A-	2111773	19-06-94
		CA-A-	2111774	19-06-94
		CA-A-	2111775	19-06-94
		DE-A-	4305552	23-06-94
		DE-A-	4305554	23-06-94
		EP-A-	0620000	19-10-94
		EP-A-	0604806	06-07-94
		JP-A-	6316553	15-11-94
		JP-A-	7002676	06-01-95
		JP-A-	6279467	04-10-94
DE-A-2756866	21-06-79	NONE		
GB-A-2045612	05-11-80	NONE		